In The Name Of God

Polymeric Engineering of Nanoparticles for Highly Efficient Multifunctional Drug Delivery Systems

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Abstract

- Developed a multifunctionalized DDs that combines high specificity towards cancer cells with endosomal escape capabilities
- The polymeric engineering here developed enhances the performance of DDS at low drug dose, holding great potential for anticancer therapeutic applications.

Introduction

Why Mesoporous Silica Nanoparticles?!!
(MSNP)

- Biocompatibility
- High loading capacity
- Chemical stability
- Straightforward synthesis/surface functionalization

Have not been translocated into the clinical stage yet!!

To promote the specific internalization of nanoparticles to certain cancer cells;

Employment of specific ligands, which can bind to receptors overexpressed in tumor cells and trigger particle internalization via endocytosis

Hyaluronic acid & Glycoprotein receptor overexpressed in many solid tumor cells

(HA) ⟷ (CD44)

- External materials taken up via endocytosis
- Normally sorted out in Endocytic vesicles (endosomes & lysosomes)
- Ejected to the extracellular matrix via Exocytosis

What is the problem ????

Non-Coated MSNPs co-localize with the endo-/lysosomes in the early stage of incubation, & are quickly exocytosed

HA-coated MSNPs are internalized via CD44-mediated Endocytosis & are subjected to same endocytic system, ending up in the acidic cellular compartments within few hours of incubation, & being ejected via exocytosis within 48 h

Problems in DDs

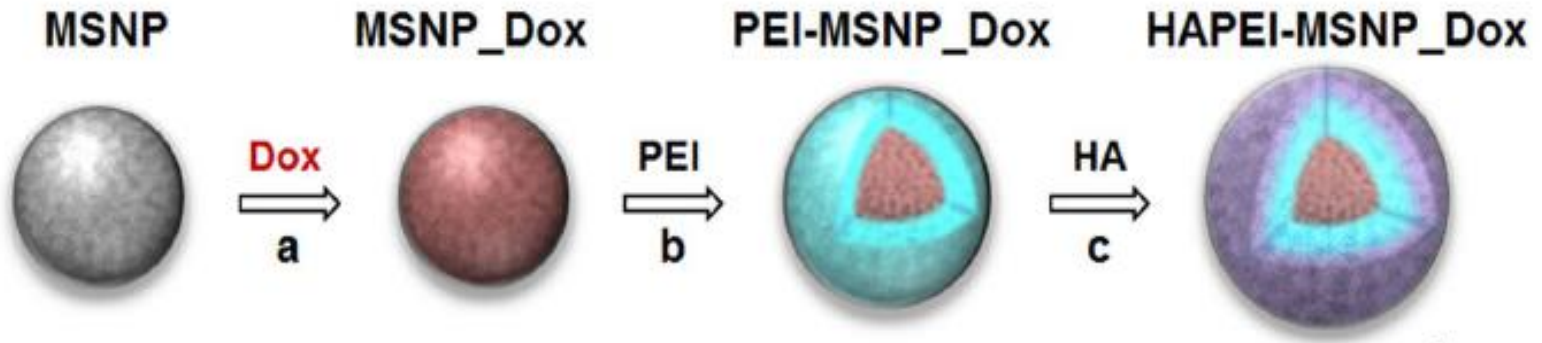
- The Endo-/Exocytosis process
- The Low lysosomal pH (4.5–5.5 for normal cells and 3.5–5 for cancer cells)
- The strong Enzymatic activity
- The Fast exocytosis of the Nanocarriers
- The DDS can be exocytosed to the extracellular matrix before releasing all its cargo

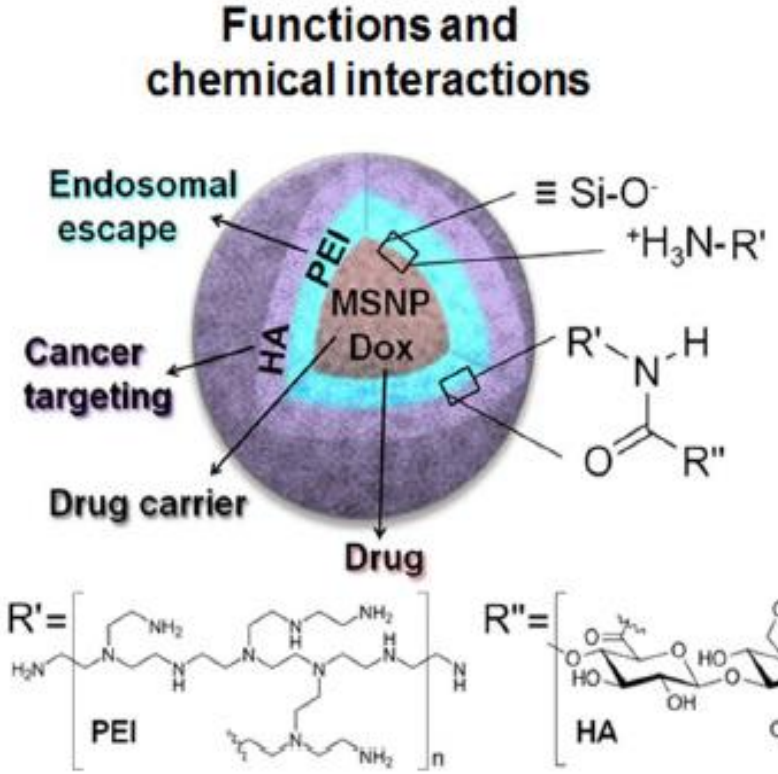
So:

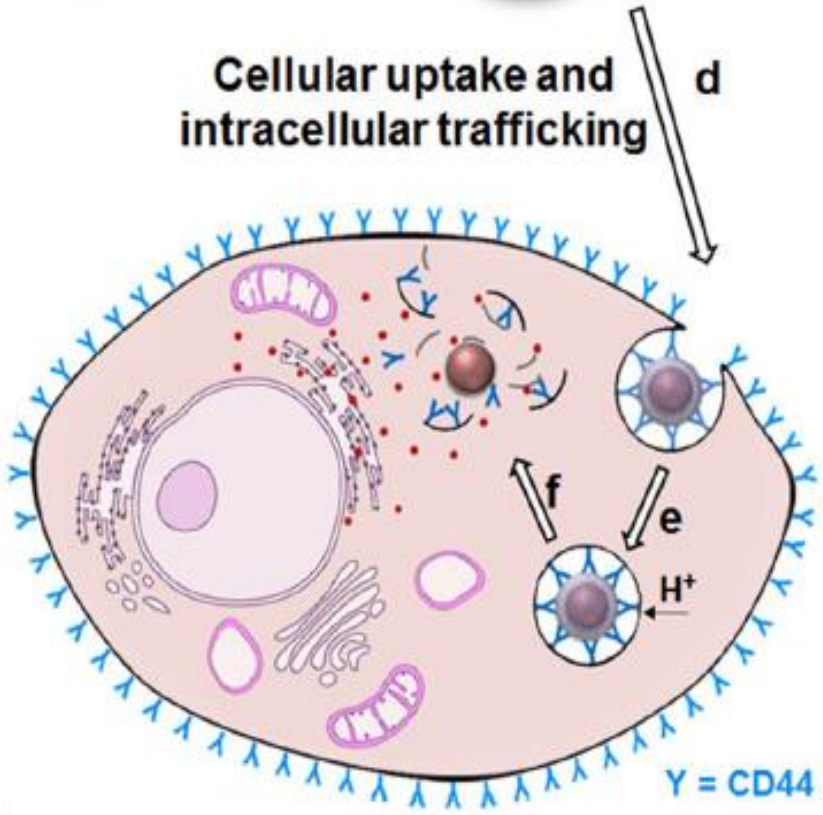
The employment of Cationic Polymers, in particular Polyethylenimine (PEI), is a promising strategy, as it is non-immunogenic and easier to scale up, compared to other agents, such as synthetic fusogenic peptides.

&

"Proton Sponge Effect" of PEI


MSNP
MSNP_Dox
PEI-MSNP_Dox
HAPEI-MSNP_Dox
Dox
a
PEI
b
HA
c


Functions and
chemical interactions
Endosomal
escape
PEI
MSNP
Dox
HA
Cancer
targeting
Drug carrier
Drug
≡ Si-O⁻
⁺H₃N-R'
R'
N
H
O
R"
R'=
PEI
R"=
HA


Cellular uptake and
intracellular trafficking
d
f
e
H⁺
Y = CD44

Assume

- the Endosomolytic activity of the PEI layer
- the addition of a PEI layer can slow down the Exocytosis rate of MSNPs

✓ A facile method to functionalize MSNPs with a polymeric bilayer, which simultaneously combines the active targeting action of HA & PEI-mediated endosomal escape

Material

Materials. Tetraethyl orthosilicate (TEOS, 98%), cetyltrimethylammonium chloride solution (CTAC, 25% in H_2O), triethanolamine (TEA, 99%), hydrochloric acid (HCl, 1 N), rhodamine B basic violet 10 (RhodB, 93%), fluorescein 5(6)-isothiocyanate (FITC, ≥90% HPLC), polyethyleneimine solution (PEI, 50% w/v in H_2O), N-(3-dimethylaminopropyl)-N′-ethyl-carbodiimide (EDC, 97%), N-hydroxysulfosuccinimide sodium salt (sulfo-NHS, ≥98% HPLC), doxorubicin hydrochloride (Dox, suitable for fluorescence, 98–102%, HPLC), sodium acetate buffer solution, MES hydrate (titration, ≥99.5%), hyaluronidase type I-S (Hyal-1, from bovine testes), hyaluronidase Type II (Hyal-2, from sheep testes) were purchased from Sigma Aldrich. Sodium hyaluronate (HA, research grade, 289 kDa) was obtained from LifeCore BioMedical. Dulbecco's modified eagle medium (DMEM), and Lysotracker RED DND-99 were purchased from Molecular Probes. Gentamicin, Dulbecco's phosphate buffered saline (PBS, no calcium, no magnesium), Hank's balanced salt solution (HBSS, no phenol red), GlutaMaxi supplement, fetal bovine serum (FBS, South America origin), Ethanol (absolute, 99.9%), Vybrant DiO cell-labeling solution were purchased from ThermoFisher Scientific. Trypan blue solution (0.4%, TC grade) was purchased from Life Science. All the chemicals were used without further purifications.

Methods:

1- HAPEI-MSNps Preparation:

synthesis of **MSNPs** → The loading of **RhodB** and **FITC** →

MSNPs dispersed in phosphate buffer (pH 9) to increase the loading efficiency

↓

the **Dox** soaking → mixtures under magnetic stirring (500 rpm) overnight

= **MSNPs_RhodB/FITC/Dox**

PEI in H2O at pH 7 and added to loaded-MSNPs in milli-Q water → about 3 h under magnetic stirring → **HA** were dissolved in 10 mL of MES buffer → stirring for 30 minutes for the activation of the carboxylic groups → carboxyl-activated HA added to 3 mL of PEI-MSNPs_X

= **HAPEI-MSNPs_X**

- Analyzed with UV-VIS spectrometer in order to estimate the soaked cargo concentrations via absorbance

Methods

HAPEI-MSNps - X characterization:

- transmission electron microscopy (TEM): for size, shape & porosity
- collecting wide-field images and the relative emission spectrum of MSNPs_Dox/RhodB/ FITC: The loading
- inverted optical microscope (TiU, Nikon): measurements
- Nd:YAG laser : applied on RhodB samples

Methods

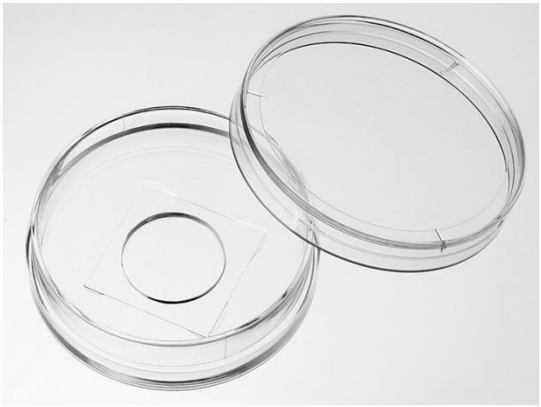
Cellular uptake: NIH3T3 and A549 cell lines

All cells → **at 37 °C, 5% CO2, in 25 cm2 cell culture flasks** → **the confluency reached 80%** → in DMEM medium

containing 10% FBS, 1% L-glutammax and 0.1% gentamicin

Then

cultured in 35-mm glass bottom dishes for confocal imaging



- (NIH3T3 cell lines: Embryonic fibroblast cells)
- (A549 cell lines: Adenocarcinomic human alveolar basal epithelial cells)

Methods

Cellular uptake:

For verifying the effect of different functionalization on the uptake;

RhodB-loaded particles (MSNPs_RhodB, PEI-MSNPs_RhodB &HAPEI-MSNPs_RhodB)

I. incubated with NIH3T3 and A549 cells
II. plasma membrane was stained with DiO
III. visualized under a Confocal Fluorescence Microscope
IV. Fluorescence intensity analysis was performed by MATLAB software

Methods

Intracellular trafficking: fluorescence microscopy

- ✓ FITC : In order to track the intracellular fate of the particles (MSNPs_FITC, PEI-MSNPs_FITC, HAPEI-MSNPs_FITC) with A549 cells for 3 h.
- ✓ Washed with PBS three times : remove the residual extracellular particles
- ✓ Confocal fluorescence measurements after the washing (data of 3 h of incubation)

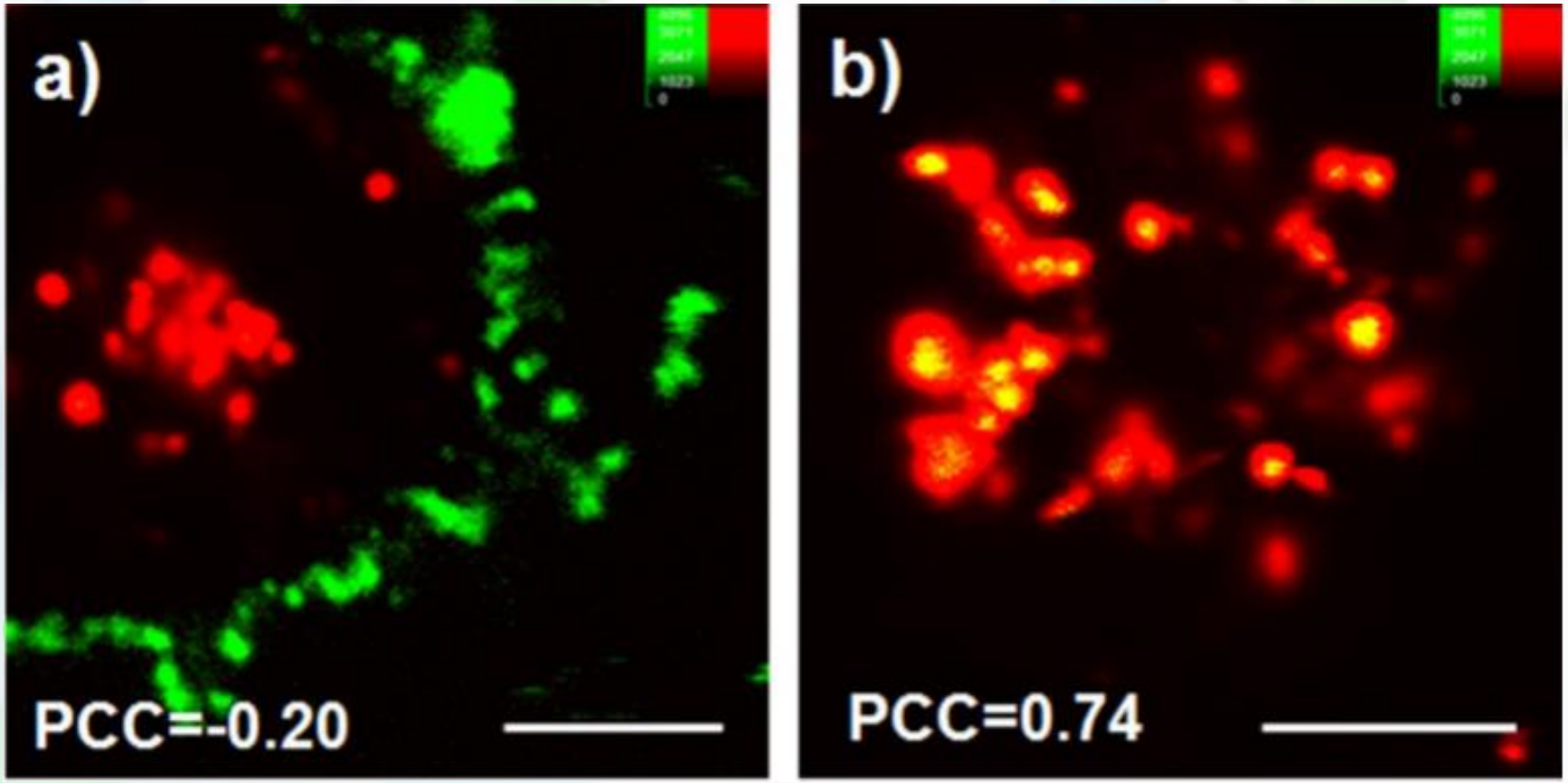
(other copies of the same samples in fresh medium and placed back in the incubator for measurements after 24 and 48 h)

- ✓ stained with Lysotracker Red: For the acidic compartment imaging, endo/ lysosomes
- ✓ Images were processed using FV10-ASW Viewer Software

- Pearson correlation coefficient (PCC) analysis by using MATLAB software

PCC values	indicate that:
Near 0	the fluorescence intensities of the two channels are uncorrelated
Close to 1	the two fluorescence intensities are perfectly linearly related

$$PCC = \frac{\sum_i (R_i - \bar{R}) \cdot (G_i - \bar{G})}{\sqrt{\sum_i (R_i - \bar{R})^2 \cdot \sum_i (G_i - \bar{G})^2}},$$


a)
PCC=-0.20
b)
PCC=0.74

Methods

Drug release in vitro:

1) 3 different pH buffers: Acetate buffer (pH 4.5), MES buffer (pH 6) and PBS (pH 7.4)
2) Add Dox-incorporated particles
3) Hyal-1 and Hyal-2 add to HAPEI-MSNPs_Dox in acetate and MES buffer for…
4) at different incubation time (0–72 h),
5) Estimate the concentration of the Dox released by collecting the absorbance at 490 nm.

Methods

Drug release in Cellulo.:

10 µL of HAPEI-MSNPs_Dox + 1 mL of medium containing A549 cells

✓ **in the incubator for 3 h**

taining A549 cells on a glass-bottom dish. The dish was then placed in the incubator for 3 h. Subsequently, the un-internalized particles were washed away with PBS washing (x3) and the cells were measured by using a confocal microscope (FV 1000 Olympus microscope) without any staining, obtaining data of 3 h of incubation. On the other hand, after the PBS washing, other copies of the same samples were suspended in fresh medium and placed back in the incubator for measurements after 24 and 48 h. Differential interference contrast (DIC) images were collected to visualize the cells and Dox emission was detected by using a 488 nm laser (5 µW, power density ~5.3 kW/cm^2), a 100x oil objective (N.A. 1.4) and a bandpass filter 600–670 nm.

Methods

Anticancer efficiency:

Carry out viability tests;

I. Seed the cells with 1 mL of medium

II. aliquots of 2, 4, 6, 8 and 10 µL solutions containing HAPEI-MSNPs_Dox, free Dox & empty HAPEI-MSNPs (Dox= 80, 160, 240, 320 and 400 nM & particles= 20, 40, 60, 80, 100 µg/mL)

III. Incubated overnight, wash & …

IV. The viability was estimated: after the treatment (HAPEI-MSNPs_Dox, free Dox and empty particles) with a control dish

V. KOVA system protocol

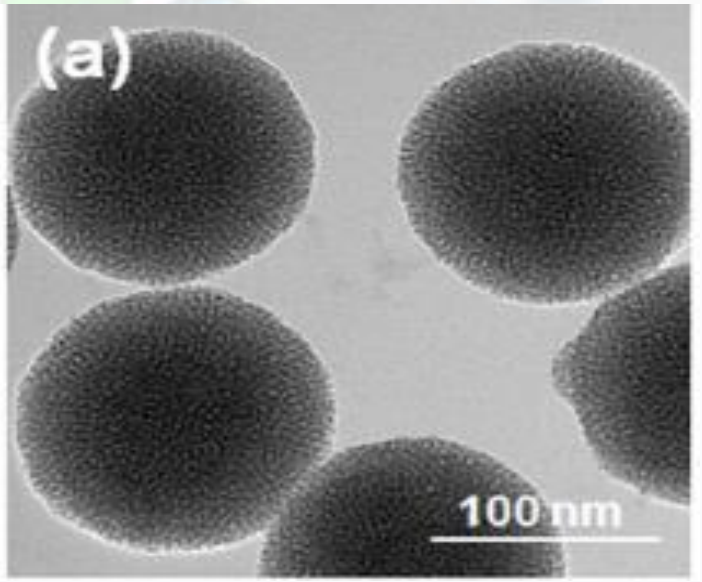
Results & Discussion

Preparation and characterization of multifunctional MSNPs.:

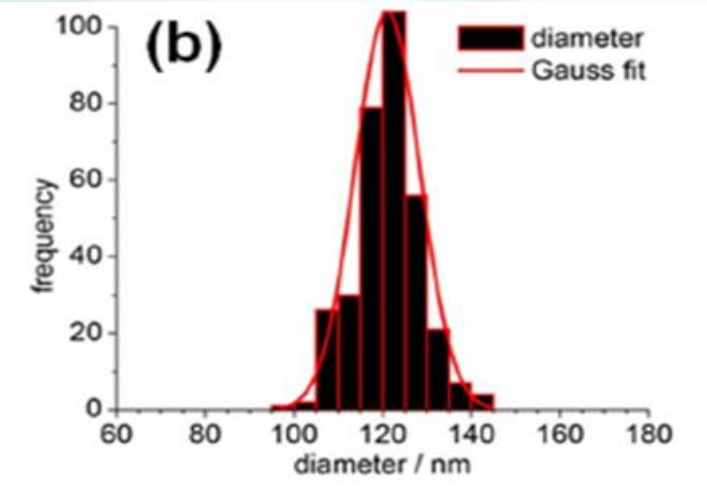
- ✓ Synthetize MSNPs using the biphase stratification method that particles with a pore size of ~2.8 nm

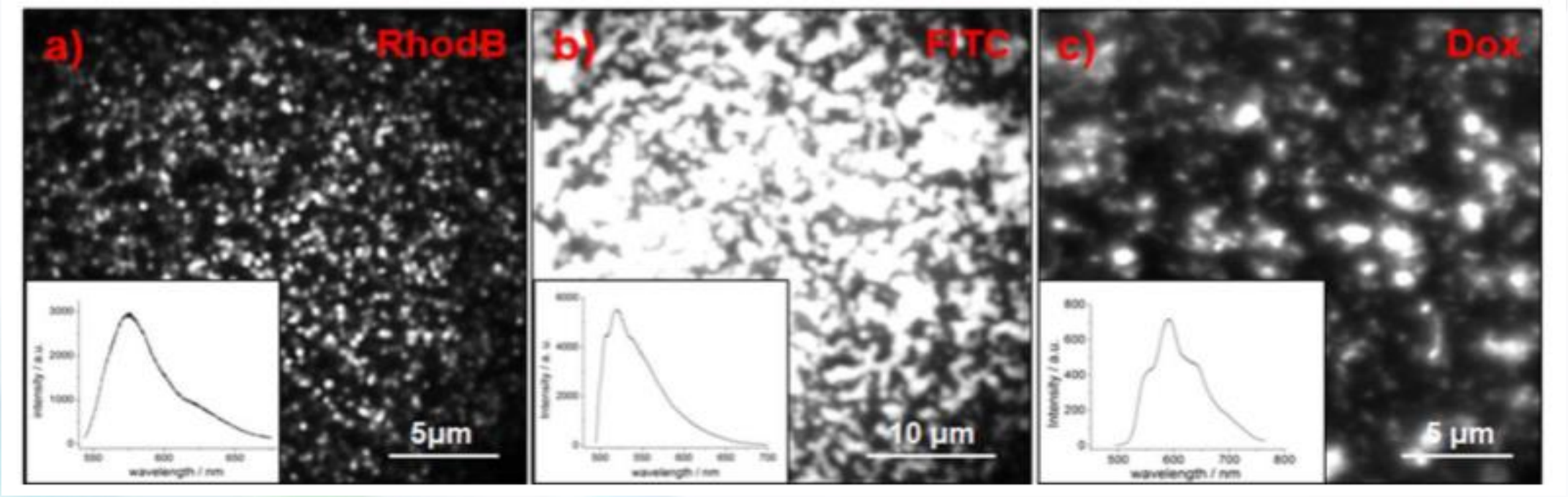
- ✓ Properties: size (120nm) & shape homogeneity,

No aggregates & uniform mesoporous frame



TEM images of uncoated MSNPs

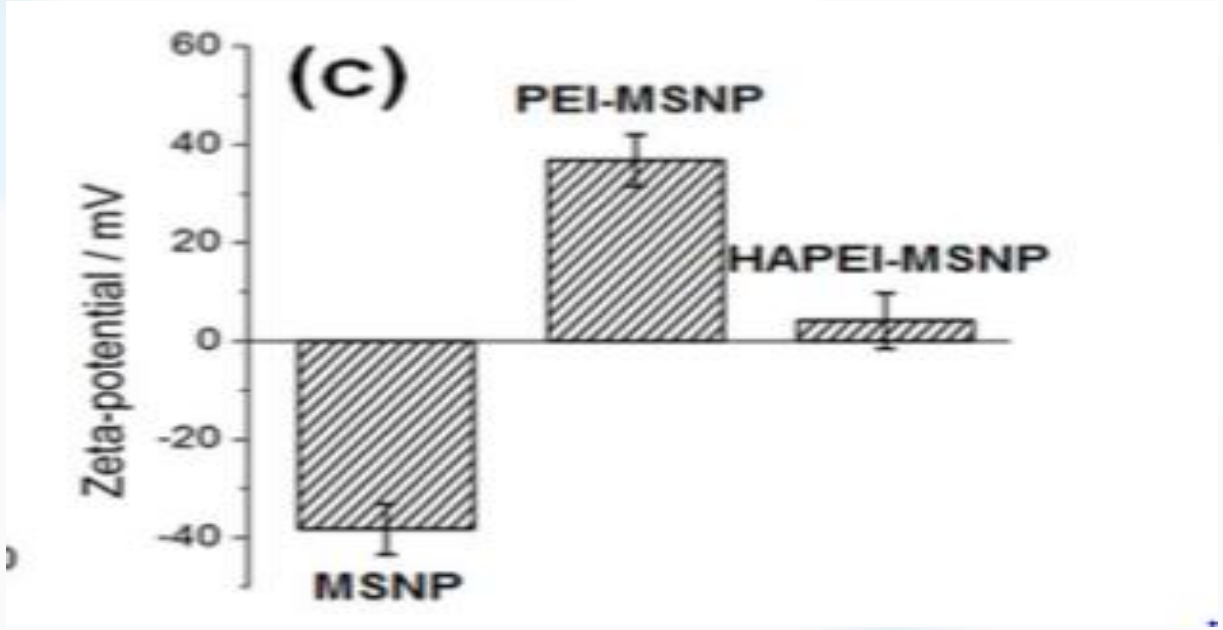


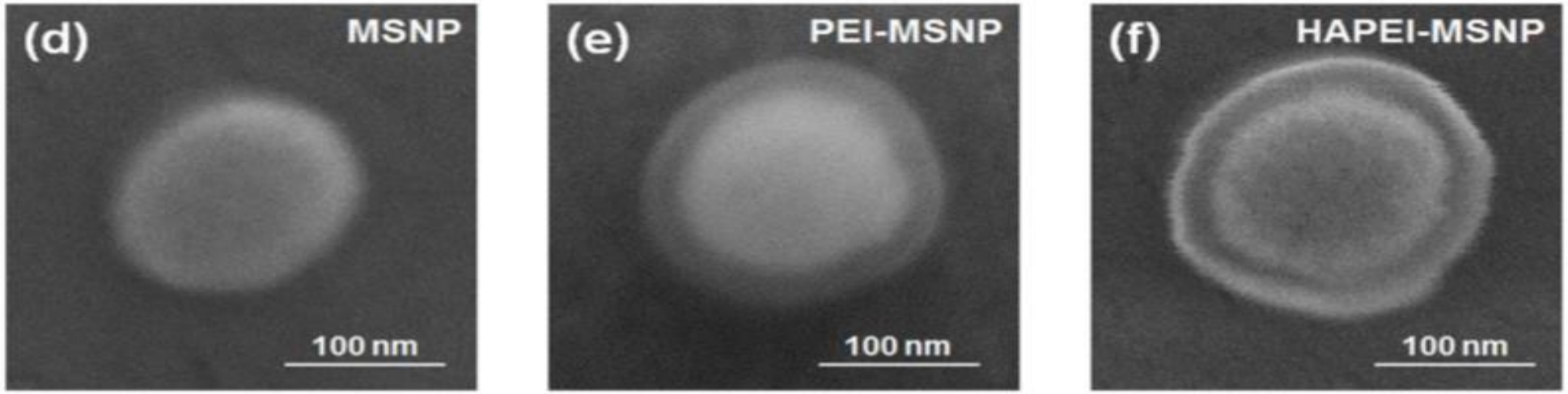


Cargo loading into MSNPs: wide-field fluorescence images (a-c) and relative emission spectra (insets) of MSNPs loaded with RhodB (a), FITC (b) and Dox (c). Emission spectra were collected by focusing the laser on one bright spot.

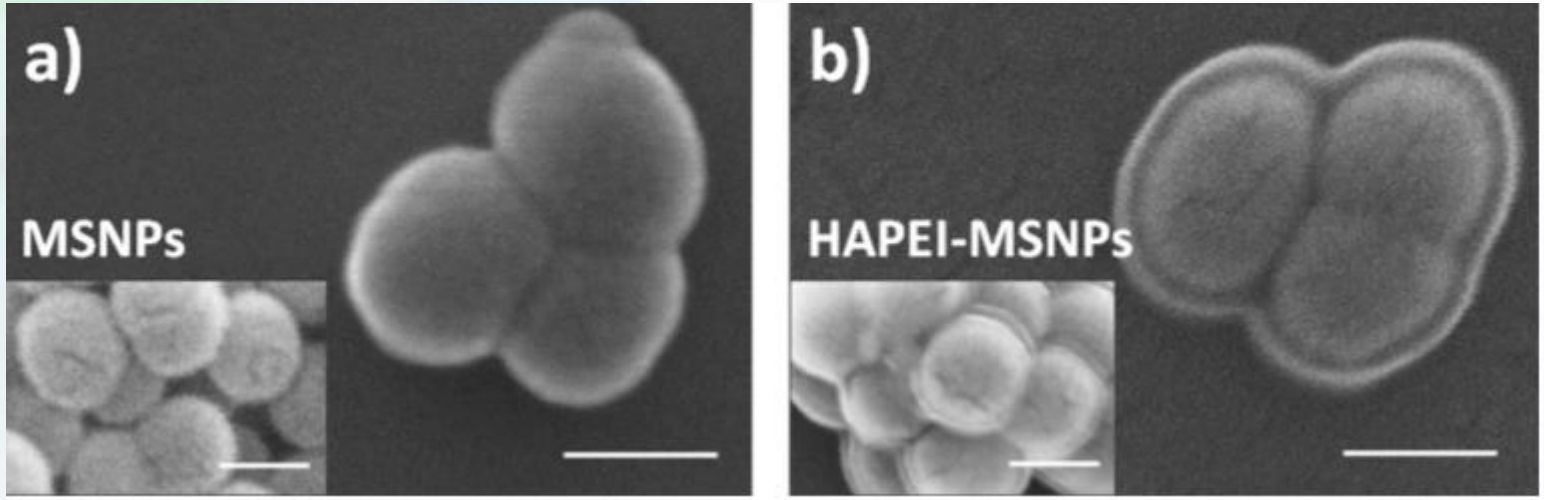
Results & Discussion

- MSNPs were coated with PEI for providing the DDS with endosomal escape capability
- ionization percentage enables the formation of a PEI shell on the MSNP surface via electrostatic interaction. The presence of the PEI layer on MSNPs was demonstrated by the drastic change in the zeta potential of the particles after the coating





after conjugation with HA, the edge contrast increases, The halo displayed in Fig. e,f was never observed for bare MSNPs (FE-SEM images)



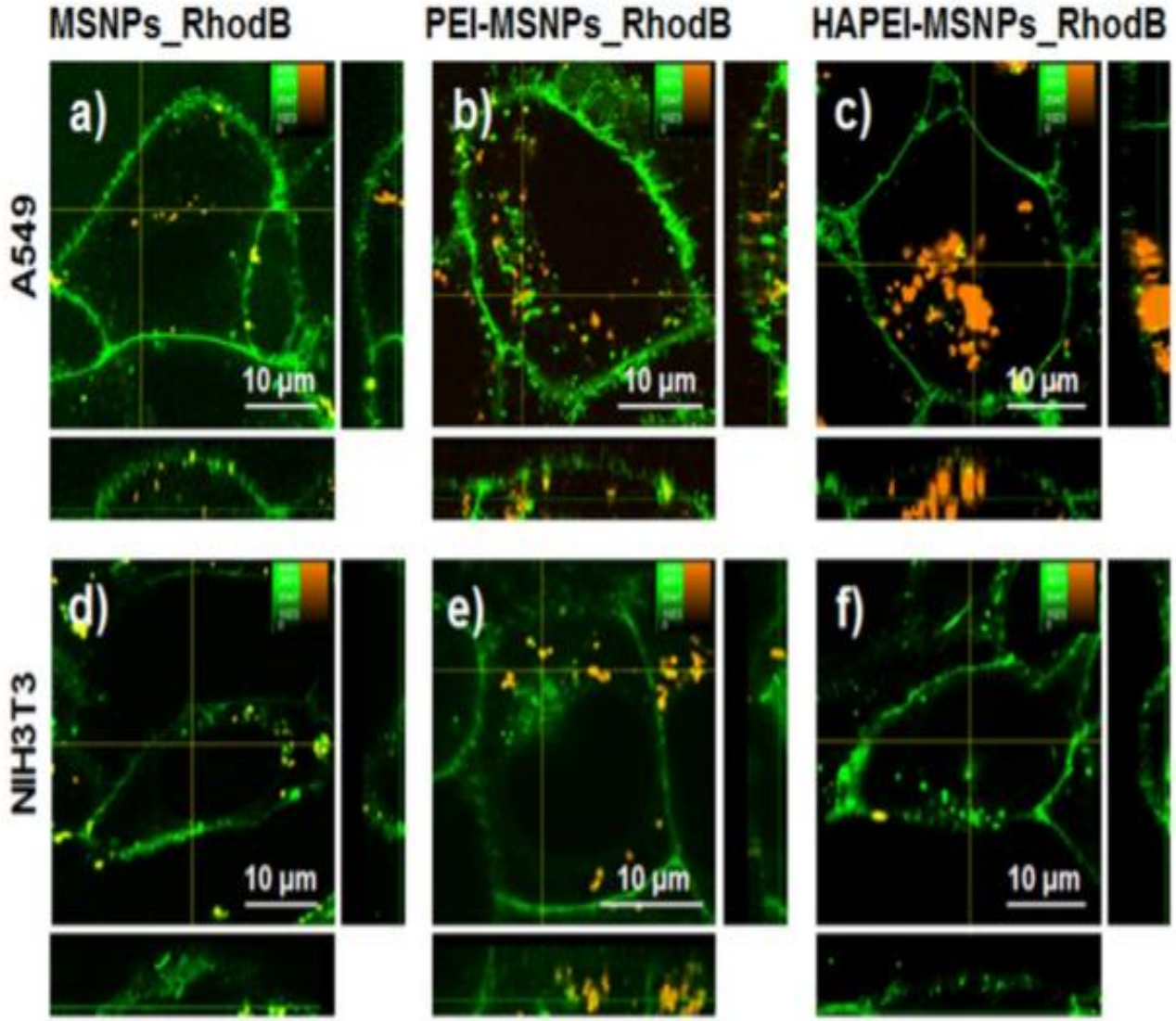
Results & Discussion

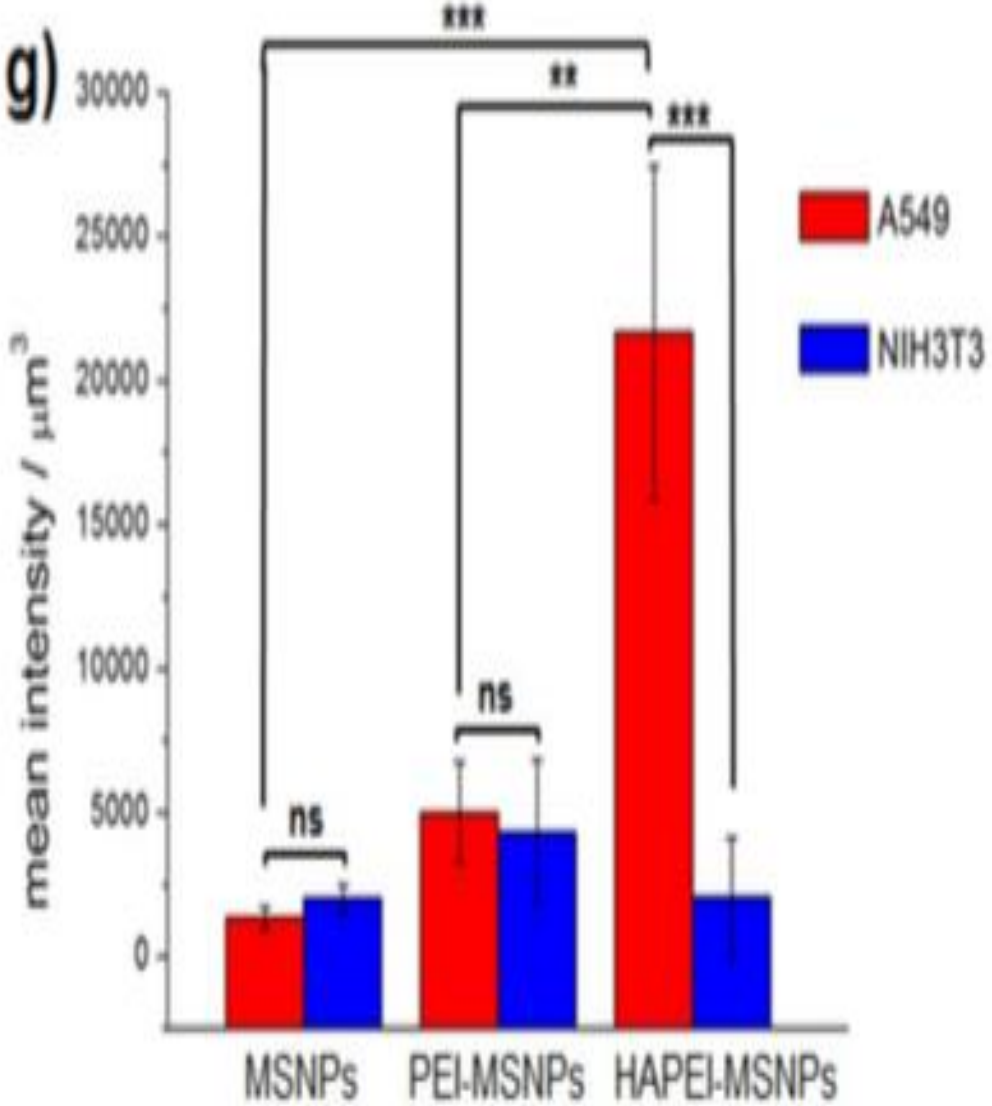
Cellular uptake: HA-mediated active targeting;

- Different particles into two mammalian cell lines for targeting efficiency & the cell specificity of the external functionalization with HA

- RhodB-loaded MSNPs with different coatings + A549 cells (CD44-overexpressing cells)

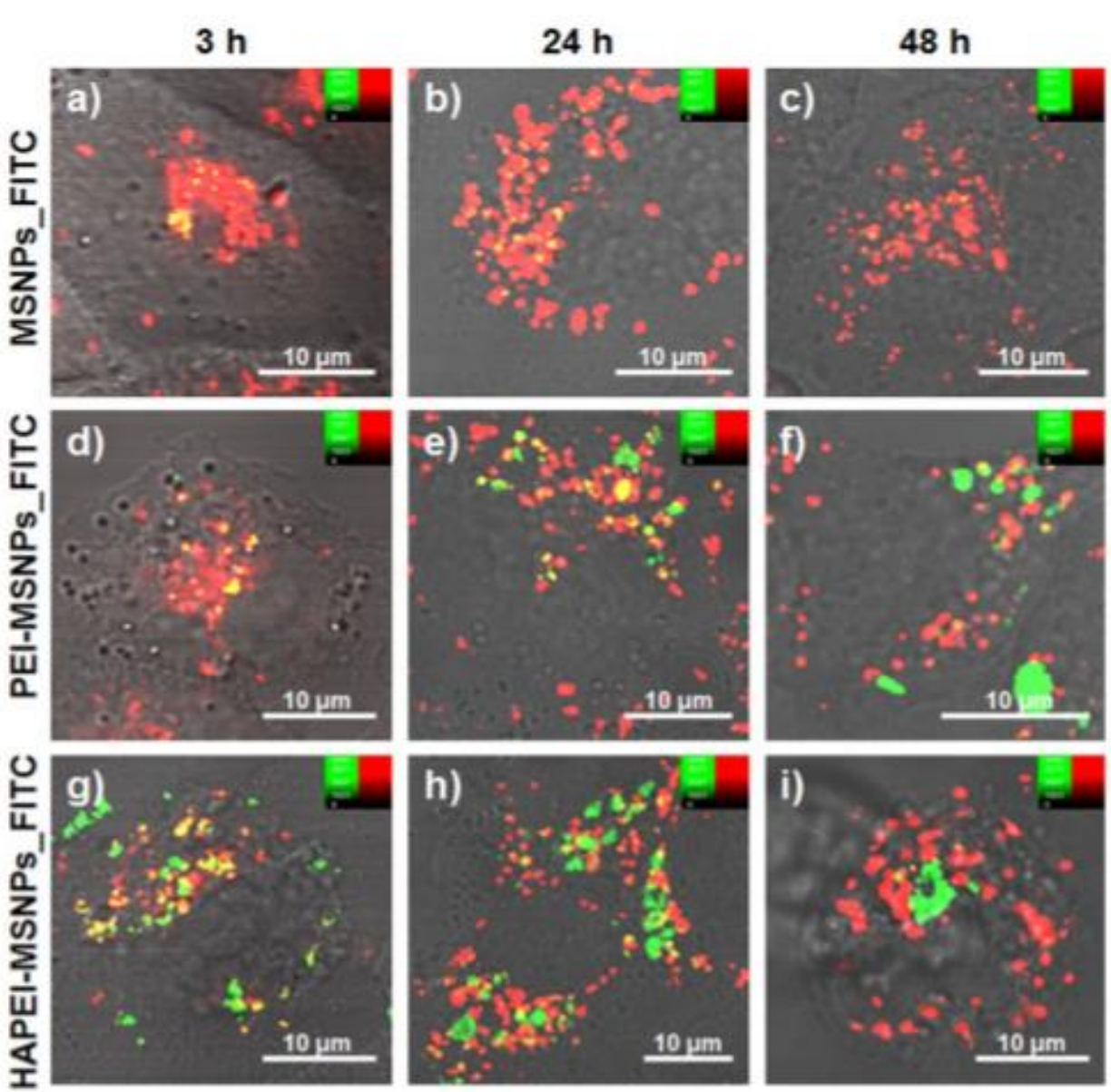
& NIH3T3 (CD44-inactive cells)

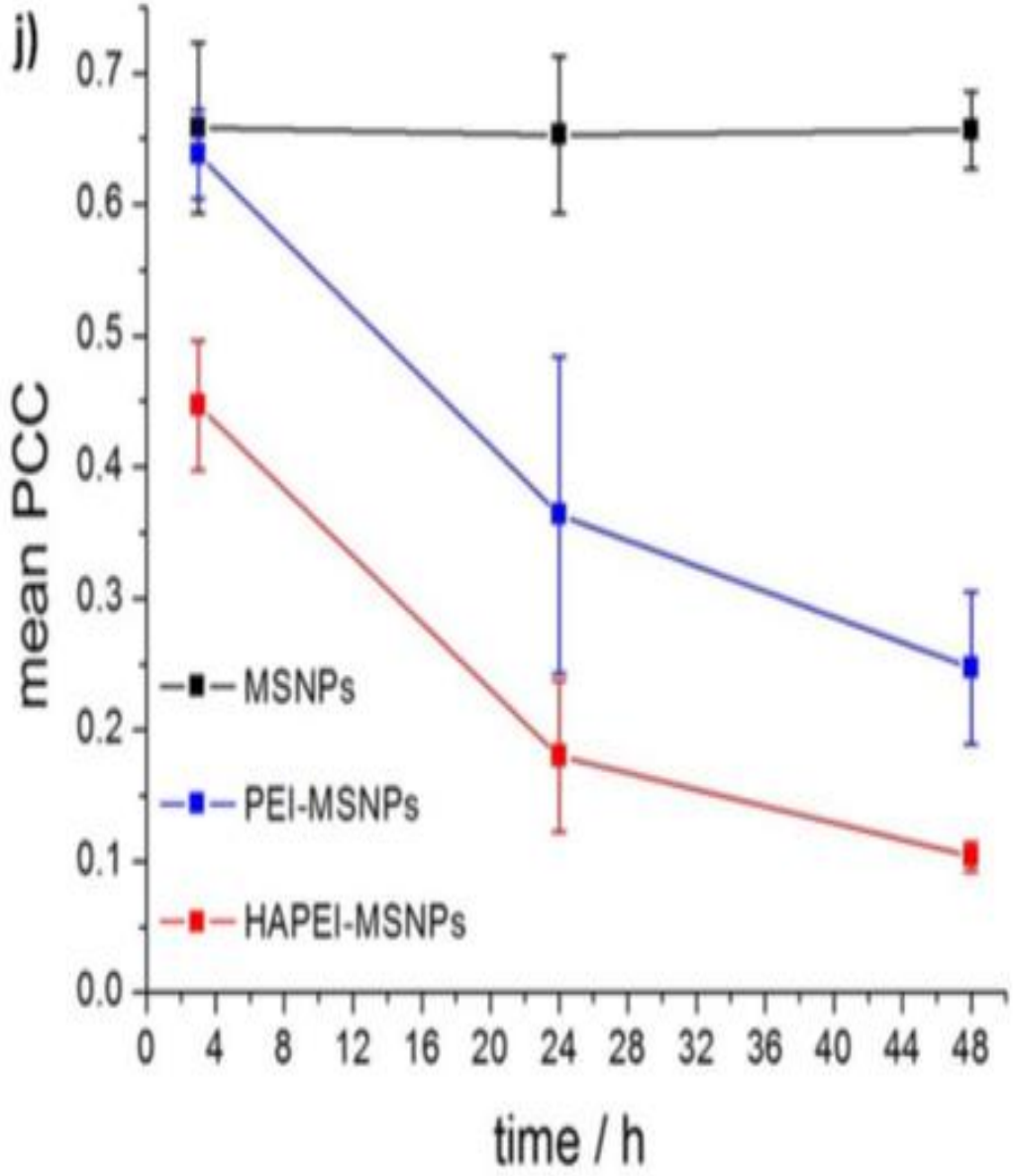




Influence of surface modification on the cellular uptake of MSNPs.

(Fluorescence images)

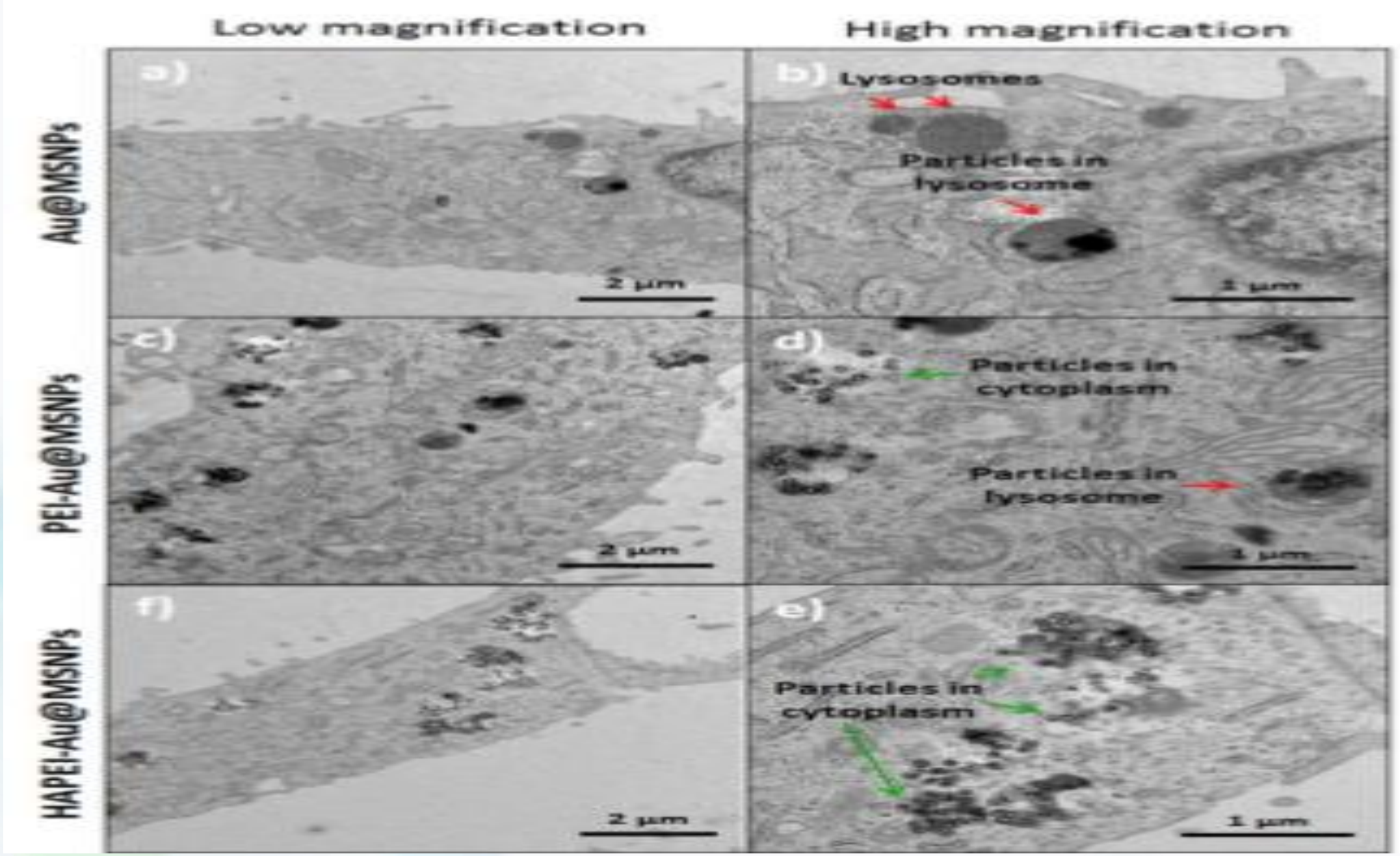




Intracellular trafficking: PEI-induced endosomal rupture

Results & Discussion

- coating of nanoparticles with specific polymers ↑ the rupture of the endo-/lysosomes and further escape to the cytosol
- Both fluorescence and electron microscopy images demonstrate an ↑ of the endosomal escape efficiency with HA-PEI coating compared to using PEI alone
- At low pH, the inclusion of an extra polymeric layer can, ↑ both the buffering capacity and the polymeric swelling, contributing to the destabilization of the endo-/lysosomal membrane.


Low magnification
High magnification
Au@MSNPs
PEI-Au@MSNPs
HAPEI-Au@MSNPs
a)
b)
Lysosomes
Particles in lysosome
2 µm
1 µm
c)
d)
Particles in cytoplasm
Particles in lysosome
2 µm
1 µm
f)
e)
Particles in cytoplasm
2 µm
1 µm

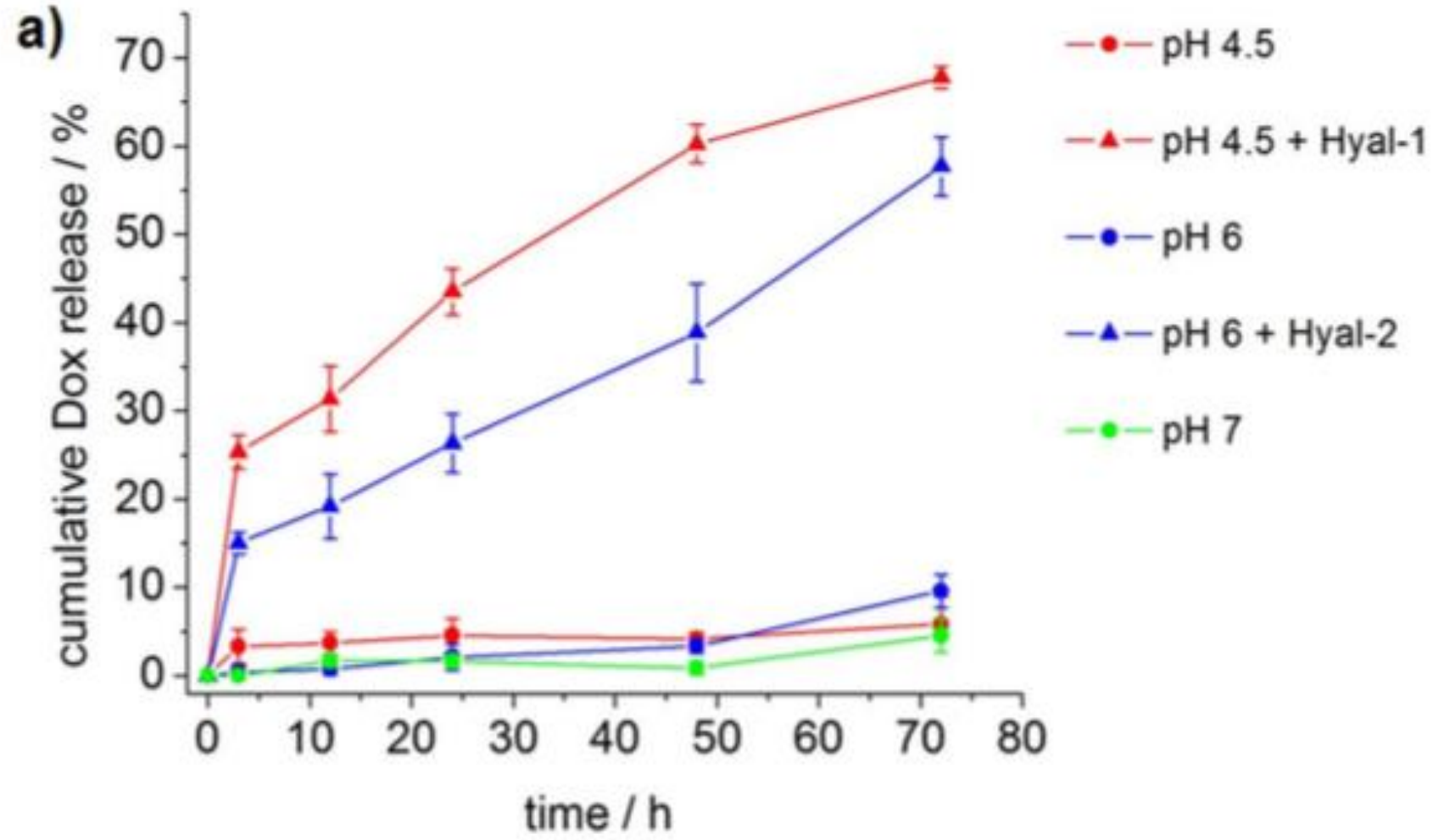
Results & Discussion

Drug release in vitro:

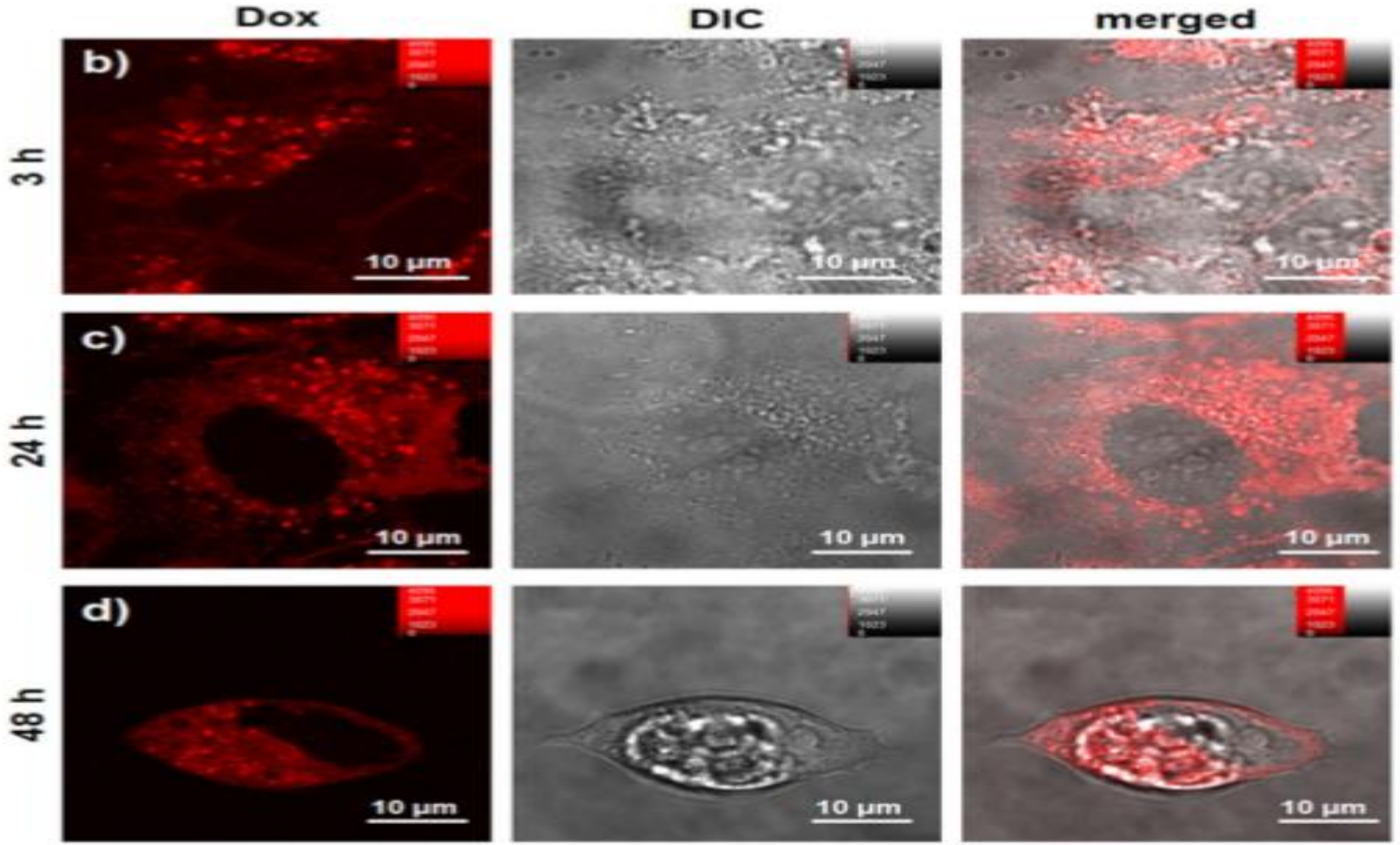
- Doxorubicin for therapeutic effectiveness & fluorescent properties
- DDSs should combined with a controlled release at the specific target.
- bare MSNPs, the environmental pH plays a crucial role on the drug release kinetics. the release rate of Dox in vitro is ✚ at acidic pH, although a relatively smaller amount can be released at neutral pH as well.

Results & Discussion

- the HA-PEI polymeric bilayer will function as a capping agent, preventing the leakage of the drug before reaching the intracellular environment
- At neutral pH, according to the pKa values of PEI and silica hydroxyl groups, the electrostatic interactions guarantee a stable attachment of the PEI shell to the particles, ⊘ the discharge of Dox in blood circulation
- At acidic pH, as the majority of the hydroxyl groups of the silica particles are protonated, the electrostatic interactions are minimized, ↓ the capping effect of the polymeric coating and ↑ the drug release in the cellular acidic compartments



only enzyme-mediated degradation of the polymeric coating, which occurs exclusively in the cellular environment, triggers drug release from the particles



Drug release in Cellulo.:

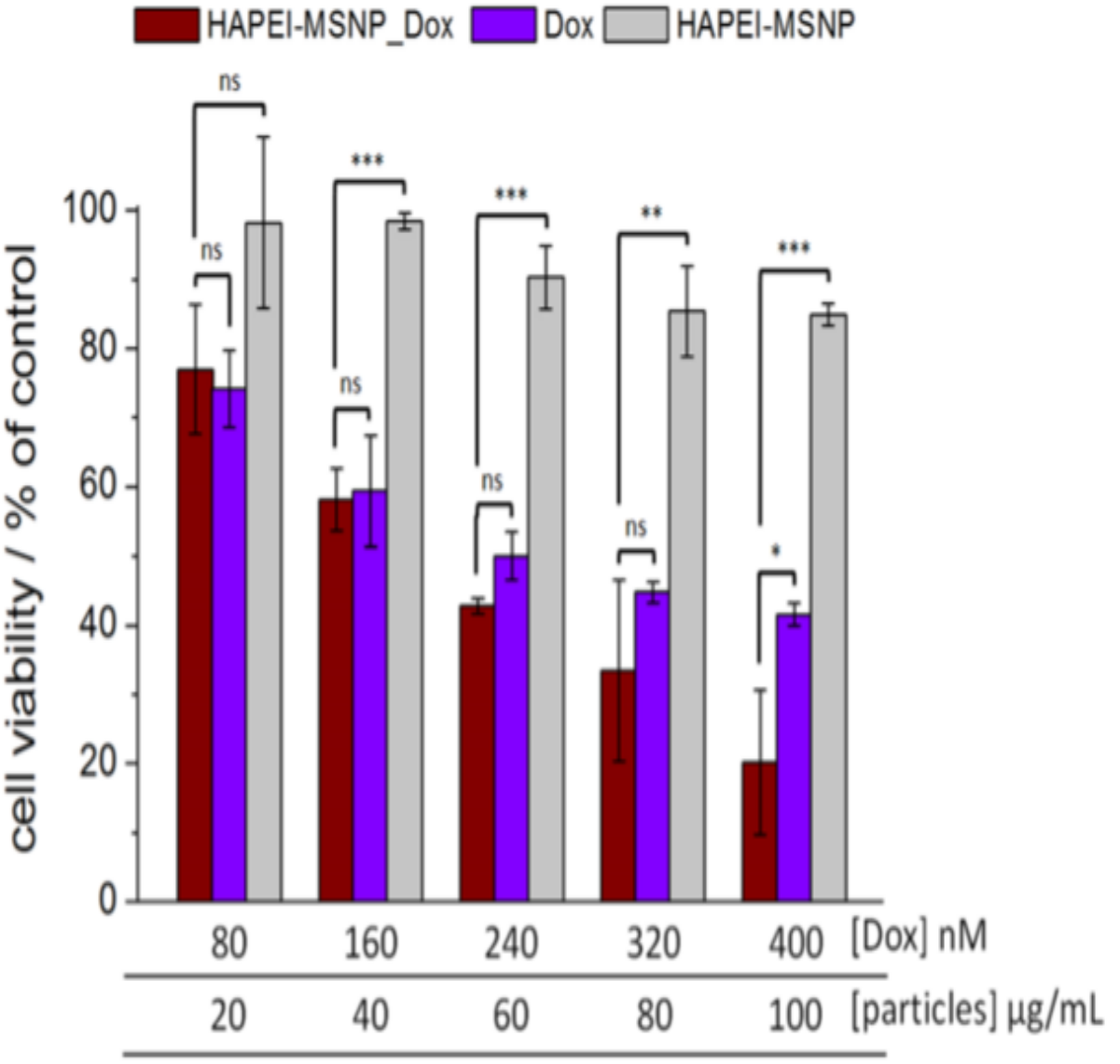
Fluorescence images of Dox released from HAPEI-MSNPs_Dox inside A549 cells after 3, 24 and 48 h

Results & Discussion

Anticancer Efficiency: cell viability tests:

In order to evaluate the efficiency polymer-coated particles as anticancer DDSs, cell viability 72 h after treatment with free Dox, Dox-loaded HAPEI-MSNPs & empty HAPEI-MSNPs, at different concentration of drug/particles

- (high uptake, endosomal escape & controlled drug released)



Results & Discussion

- our DDS has great therapeutic potential, specifically towards CD44-overexpressing cancer cells
- The high therapy efficiency achieved at low drug concentrations is strictly related to:

- the fast internalization rate (HA coating targeting effect)
- to the improved endosomal release (with consequent retention of the particles in the cytoplasm ,HA-PEI shell inducing endosomal rupture),
- control drug release overtime (enzymatic polymeric digestion)

Results & Discussion

1) HA & PEI layers provide the DDs with active targeting & endosomal escape capability, enhancing the therapeutic efficiency
2) Dox-loaded HAPEI-MSNPs exhibit great killing efficiency at low drug concentrations, which was comparable with that of pure Dox, but with specificity towards CD44-overexpressing cancer cells

Great potential DDs for tumor therapy applications ;

- ✓ endosomal escape capacity
- ✓ controlled drug release
- ✓ Consequently
- ✓ high therapeutic effect
- ✓ targeting capability towards cancer cells

Future Perspective

- The polymeric functionalization proposed can be applied to a wide range of nanocarriers towards the increase of the therapeutic power at low drug dose and the decrease of exocytosis rate, drastically reducing the side effects of anti-cancer drugs.


Change your thoughts & you change your world.
thanks